SCHEDULE-DEPENDENT SYNERGY AND ANTAGONISM BETWEEN CYTOSINE ARABINOSIDE AND L-ASPARAGINASE ON THE L5178Y MURINE LEUKEMIA

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SCHEDULE-DEPENDENT SYNERGY AND ANTAGONISM BETWEEN CYTOSINE ARABINOSIDE AND L-ASPARAGINASE ON THE L5178Y MURINE LEUKEMIA

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Abstract

Treatment of L5178Y *in vitro* cells with ara-c (10^{-6} M) or asparaginase (0.5 IU/ml) for 8 hours resulted in 45% and 24% viability, respectively; simultaneous exposure to both drugs resulted in 25% viability, a subadditive effect. Sequential 8 hour treatments with asparaginase preceding ara-c or ara-c preceding asparaginase resulted in 43% and 8% viability, respectively, indicating strong schedule-dependency. While ara-c pretreatment resulted in cell synchronization it did not enhance asparaginase-induced inhibition of DNA and protein synthesis. *In vivo* recovery from drug-induced inhibition of cell growth suggested an optimal interval of 120 hours. Mice were inoculated with 10^6 cells. Treatment with asparaginase, ara-c or both drugs on day 3 resulted in mean survival times (MST) of 16, 21 and 18 days, respectively (control MST 10 days). With a 120 hour interval between the 2 drugs, asparaginase preceding ara-c resulted in a MST of 23 days; ara-c preceding asparaginase produced 20/24 60 day survivors. Maximal weight loss was only 10%. Mechanisms for the antagonism include asparaginase-induced decreased transport and incorporation of ara-c into macromolecules and cytokinetic mistiming. Unequivocal understanding of the apparent synergy is not adequately explained by the observed AC-induced synchronization. Since both drugs are likely components of antileukemic combinations, understanding of such drug-drug interactions would optimize clinical therapy.

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Introduction

A variety of drug interactions, ranging from antagonism to synergism, can result from the combination of two cancer chemotherapeutic agents. There is a growing interest in understanding the basis of these interactions since various studies have indicated the superiority of combination therapy for the treatment of acute leukemia (34, 40, 46, 60, 114, 125, 150). Two agents that have been studied for remission induction in acute lymphocytic and acute myelogenous leukemia are cytosine arabinoside (ara-c) and L-asparaginase (9, 54, 112, 144). Even though their combined use has produced encouraging results in the therapy of acute lymphocytic leukemia (46, 60, 114, 125), few studies have attempted to determine the optimal schedule of administration or to define the nature of their interactions.

An aim in designing combination chemotherapy is to select agents which will maximize tumor cell kill while minimizing host toxicity. This can be accomplished with agents like ara-c and asparaginase, which have different host toxicities (9, 54, 101). This will work, of course, provided that their interaction does not antagonize their antitumor activity as, for example, asparaginase antagonizing the cytotoxicity of another chemotherapeutic agent, methotrexate (22, 23, 25). Capizzi et al. (22, 23, 25) demonstrated

both schedule dependent synergism and antagonism for the combination of asparaginase and methotrexate. In brief, when asparaginase preceded methotrexate by less than 24 hours, the effect of methotrexate on murine leukemia L5178Y cells was diminished. However, when the reverse combination was tried, there was no antagonism. In fact, asparaginase protects mice against methotrexate-induced intestinal lesions when asparaginase followed methotrexate. From this work it can be concluded that there is a synergistic antitumor schedule that also minimizes host toxicity.

Although methotrexate and ara-c have different intracellular mechanisms of action (22, 25, 55, 75, 82, 165) they are both active agents in inhibiting DNA synthesis. Therefore, the main purpose of this thesis project was to determine if the combination of ara-c and asparaginase also exhibits schedule-dependency. Studies reported in this thesis describe the effect of three different treatment schedules on animal survival and cell viability *in vitro* using the animal tumor model, murine leukemia L5178Y. In addition, in an attempt to explain the interactions observed, the effect of this combination of drugs on DNA and protein synthesis was also investigated.

Literature Review

Cytosine Arabinoside

Ara-c (cytosine arabinoside, 1-B-D arabinofuranosylcytosine, Cytosar, Cytarabine) is a pyrimidine nucleoside analog of deoxycytidine, but differing in that the sugar moiety is arabinose rather than deoxyribose (55, 102). Ara-c is of considerable clinical importance because of its effectiveness against acute leukemia--particularly the acute myelogenous form--in man (9, 54). The brief review presented here will summarize some of the aspects of ara-c's cellular uptake, intracellular mechanism of action, and clinical use.

Ara-c passively diffuses into cells, where it is rapidly phosphorylated to the active form, ara-CTP (29, 31, 79, 104). Indeed, there appears to be a relationship between the ability of human and mouse leukemic cells to retain ara-c in its phosphorylated form, and the susceptibility of the cells to the drug (29, 79). On the other hand, resistance of cells to ara-c has been correlated with decreased levels of deoxycytidine kinase (136), the enzyme responsible for the phosphorylation of both ara-c and deoxycytidine to their respective monophosphates (ara-CMP, dCMP) (44, 79, 104). In addition, resistance to ara-c is also associated with enhanced levels of pyrimidine nucleoside deaminase which is the enzyme that

catalyzes the conversion of ara-c to its inactive metabolite, uracil arabinoside (41, 65).

The importance of deoxycytidine kinase in ara-c metabolism has prompted the investigation of the interaction between ara-c and the enzyme's natural substrate, deoxycytidine. Since the Km of the kinase for ara-c is three-fold higher than the Km for deoxycytidine, the phosphorylation of ara-c is markedly inhibited in the presence of deoxycytidine (104, 136). This fact has been invoked to explain the ability of deoxycytidine to partially rescue ara-c treated cells (104, 136). In addition, the phosphorylated products of ara-c and deoxycytidine (dCTP, ara-CTP) have been reported to be feedback inhibitors of deoxycytidine kinase (44, 103). Although ara-CTP would appear to inhibit its own synthesis, it has, however, only a weak effect on the enzyme (103). Also, Skoog and Nordenskjold reported that the intracellular pool of dCTP following ara-c treatment is sharply decreased, implying that the inhibition of the kinase by dCTP does not block the conversion of ara-c to ara-CTP (142). Since ara-CTP has been shown to be the active form of the drug, the product of deoxycytidine kinase, ara-CMP, undergoes two additional phosphorylations which have been reported to be catalyzed by deoxycytidylate kinase and nucleoside diphosphokinase respectively (109, 152).

The subject of the mechanism of action of ara-c is a complex and confusing one. However, most theories are based on evidence that this cytidine analog interacts with the DNA synthetic machinery

of the cell. DNA replication in whole mammalian cells (55, 75, 143), tumor cells (31, 39, 70, 82, 84), and DNA viruses (20) is inhibited by ara-c. This body of evidence for DNA synthesis as a target area for the action of ara-c requires explanation at a more fundamental level. Three major lines of evidence have been pursued to approach this: 1) study of the kinetic properties of DNA polymerases; 2) studies of the incorporation of ara-c into nucleic acids; 3) studies of chromosomal damage, which may represent the morphologic manifestation of other mechanisms.

As background to the discussion of the specific effects of ara-c on DNA synthesis, let us briefly characterize the cell's different DNA polymerase enzymes. The subject of mammalian DNA polymerases including the presentation of a standard nomenclature has recently been reviewed by Bollum (11). Four distinct mammalian enzymes have been designated: α, β, γ, and mitochondrial polymerase. The α polymerase (maxi polymerase, replicase, polymerase I, and polymerase A) is a high molecular weight ($>$130,000) cytoplasmic protein and is responsible for the majority of DNA synthesis associated with replication. Consistent with this role, the enzyme concentration rises sharply in S-phase (28), it is stimulated by unwinding proteins and it may be able to use RNA pieces as an initiator (27). N-ethylmaleimide is a specific inhibitor of the α polymerase (11).

The β polymerase(mini polymerase, polymerase II, polymerase B, and repair enzyme), is a low molecular weight protein ($\approx$ 45,000)

found in both the cytoplasm and nucleus (26). Levels of this enzyme remain constant throughout the cell cycle and it has been presumed to be involved in post-replication repair (160). The γ polymerase (R DNA polymerase) accounts for a minor fraction of DNA polymerase activity ($< 1\%$) and its role in cellular replication is unknown (146). The mitochondrial polymerase also accounts for less than 1 percent of the polymerase activity and function is presumed to be limited to synthesis of mitochondrial DNA (72, 99).

Most of the research on the interaction of ara-c and DNA polymerases predates the techniques for separating these different enzymes. Therefore, hypothetical mechanisms of action of ara-c were based on studies using crude polymerase extracts.

The competitive inhibition of DNA polymerases has been proposed to explain the cytotoxicity of ara-c. Many authors have reported inhibition of crude extracts of DNA polymerases by ara-c. This inhibition was found to be due to ara-c's competition with deoxycytidine triphosphate (dCTP) as a substrate for DNA polymerases from partially purified extracts of calf thymus (52, 101) or from Walker 256 Carcinosarcoma (51), with lysates of mouse L cells (55), and mouse lymphoma cells (106) and with crude reverse transcriptase preparations from Raucher Leukemia Virus (106, 154). The Ki's for ara-c reported in these studies were reasonably low, suggesting that this cytidine analog might inhibit this reaction within intact cells. Further evidence for the competitive nature of this inhibition has been reported by Furlong (51) who demonstrated that ara-CTP did not inhibit

incorporation of thymidine triphosphate into DNA synthesized on an artificial template, poly dA-T. Since normal rules of base pairing preclude dCTP incorporation into such a molecule, and ara-CTP did not block DNA synthesis, it was concluded that ara-c's interference with polymerase function was limited to competitive binding at the dCTP site. The cause and effect relation between the competitive inhibition of the polymerase and cell death has not adequately been demonstrated.

The cytocidal effect of ara-c has been suggested to result from a state of unbalanced growth caused by inhibition of DNA synthesis while initially little effect of ara-c on RNA and protein synthesis occurs (143). Experiments reported by Graham and Whitmore (55) attempted to elucidate the connection between cell death and polymerase inhibition and to refute the theory of unbalanced growth. Studies on mouse L-cells showed that 3.6×10^{-7}M ara-c inhibited DNA synthesis by more than 97% and that these cells underwent "unbalanced growth" for periods greater than one generation (24 hours) without necessarily losing viability. However, at a concentration of 7.2×10^{-6}M for 2 hours, there was an irreversible loss of viability sufficient to kill all S-phase cells. These authors concluded that unbalanced growth was not responsible for cell death and that if massive inhibition of DNA synthesis was to be implicated, a mechanism had to be proposed whereby preservation of a relatively small amount of DNA synthesis, as little as 3% of the normal rate, can prevent loss of viability. Such a mechanism will be discussed below along with the

selective effects of ara-c on different cellular DNA polymerases.

There have been numerous reports which apparently contradict the proposal that competitive DNA polymerase inhibition is causally related to cell death. Several investigators (31, 84, 165) have demonstrated that cell damage induced by high doses of ara-c either is not reversed or only partially reversed by dCTP. An irreversible component of ara-c's action was clearly shown by Karon and Shirakawa (75). After Don C cells were treated with 10 μg/ml of ara-c for 24 hours, 80-90% of the cells remained viable as demonstrated by trypan blue exclusion. However, when these cells were washed and resuspended in drugfree medium, only 10% of the cells were viable by cloning. Therefore, despite removal of the drug, ara-c had already caused an irreversible change.

Momparler's studies on the interaction of ara-c and fluorodeoxyuridine (FUDR) raised yet more doubts as to the role of polymerase inhibition in cell death (102). Fifty percent of S-phase HeLa cells treated with 1 X 10^{-5}M ara-c for 1 hour were killed. But a subsequent 1 hour treatment with a sublethal dose of FUDR completely rescued the cells from ara-c toxicity. Since FUDR is also an S-phase specific DNA synthesis inhibitor (61), an additive effect with ara-c on cell kill and DNA synthesis was anticipated. Although the basis for the observed antagonism is unknown, this study further suggested that cytotoxicity of ara-c is not adequately explained by inhibition of DNA synthesis. These results have been confirmed by other investigators (57). In view of the poor correlation of ara-c's

cytotoxicity with its competitive inhibition of DNA polymerases, other known effects of ara-c will be explored in order to further clarify the subject.

There are several reports that indirectly support the hypothesis that the acute cytotoxic effect produced by ara-c results from its incorporation into DNA. First, H^3ara-c has been shown to be incorporated into DNA of various mammalian cells (29, 31, 37, 39, 56, 143, 166). Second, purified mammalian DNA polymerases can catalyze the incorporation of H^3ara-CTP into DNA *in vitro* (51, 100, 101). The incorporated radioactivity has been chromatographically proven to be ara-c (31, 37, 166). Third, short exposures of mammalian cell to ara-c have been shown to be mutagenic (69).

Despite such evidence that ara-c is incorporated into DNA, the mechanism of this presumed highly toxic effect needs to be clarified. One possibility is terminal incorporation of ara-c into DNA with cessation of chain growth. This could be a serious lesion and in fact this chain defect was observed by Momparler using DNA extract from calf thymus (100, 101). However, this observation was not confirmed using DNA polymerase extracts from Walker Carcinosarcoma or in whole mammalian cells (31, 56). In both systems ara-c was incorporated into internal nucleotides exclusively.

The discrepancy between these studies might be explained by the reported presence in thymus extracts of an unusual DNA polymerase, terminal transferase (10). This enzyme, found only in the thymus and leukemic cells, catalyzes the elongation of preformed oligomeric

or polymeric DNA chains by adding deoxyribonucleoside monophosphates to the 3'OH ends of these chains (53). Terminal transferase is unlike any other known DNA polymerase in that it does not use nucleic acid templates for instruction (10). Therefore, the absence of a free 3'OH group on a strand of DNA results in chain termination since the polymerase cannot skip to another region of the template and continue operations. Consequently, ara-c's lack of an available 3'OH group might cause chain termination in the terminal transferase reaction but not necessarily with other polymerases, which function with a template. One could speculate that ara-c induced chain termination in the presence of terminal transferase could be the basis for the selective toxicity of ara-c for leukemic cells. Although this hypothesis is attractive, there are no studies that demonstrate such an effect; therefore, other lines of evidence must be considered.

One could assume that if the incorporation of ara-c into DNA represents the cytotoxic lesion, the rate of incorporation would be proportional to cell lethality. Such a correlation was not observed in studies by Graham and Whitmore or Chu (30, 56). Chu demonstrated that when murine leukemia L5178Y cells were exposed to 3.3×10^{-5}M ara-c, incorporation of H^3ara-c into DNA stopped at 1 hour, although cell lethality continued to increase. However, the high dose of ara-c used could have blocked its own incorporation by inhibiting the polymerase completely. But, since the higher dose of ara-c is associated with increased cell death and the incorporation of ara-c into DNA is dependent on a functional polymerase, for which high dose ara-c

is a potent inhibitor, a new hypothesis would have to be proposed to reconcile this apparent contradiction.

The discrepancies in the above discussion could be explained by assuming that the high dose of ara-c potentiates the cytotoxicity of the ara-c already incorporated into DNA by inhibition of its excisional repair. A differential sensitivity to ara-c for the repair and replicative function of the polymerases was first suggested by Cleaver (35), who demonstrated that the repair of ultraviolet-induced lesions in HeLa cells containing DNA substituted with 5-Bromouracil was not inhibited by ara-c. There is precedent for this selectivity of ara-c in other eukaryotic and prokaryotic cells.

In E. coli, three distinct DNA polymerases have been isolated and designated: I, II and III (151). The function of polymerase I has been implied from studies of E. coli Pol Al mutant in which this enzyme is lacking (126). Since these cells replicated normally except for increased sensitivity to ulatraviolet-induced damage, polymerase I has been presumed to be responsible for DNA repair (126). Polymerase II has been proposed to be the replicase, and consistent with this function in mammalian cells, its activity is potentiated by unwinding proteins (126). Rama Reddy et al. have reported that the replicase (polymerase II) is sensitive to inhibition by ara-c while the repair enzyme (polymerase I) is resistant (126). This finding has been confirmed by others (151). A similar observation was made by Winterberger in studying the simple eukaryotic organism yeast (161). The replicase, as in E. coli, was more sensitive to ara-c than the putative repair enzyme.

The differential sensitivity of various DNA polymerases has also recently been reported in mammalian cells (45, 90, 91, 137, 149). Lynch et al. was able to separate two distinct polymerases from isolated hepatocyte nuclei. The first was a high molecular weight (7.1S) protein that showed a marked increase in concentration associated with the rise in DNA synthesis induced by partial hepatectomy or thyroid hormone infusion (90, 91). These properties are consistent with the mammalian replicase, polymerase α. Since the second hepatocyte polymerase is a low molecular weight (3.2S) protein that is able to repair single strand breaks in DNA induced by bleomycin (132), it resembles the mammalian DNA repair enzyme, polymerase β. Stenstrom et al. using isolated hepatocyte nuclei were able to demonstrate that the replicase, polymerase α, is 1000-fold more sensitive to ara-c than is the repair enzyme, polymerase β (45, 149). Lynch et al. (91) have confirmed these findings using the same system. However, when normal human lymphocytes were analyzed, the Ki of polymerase β for ara-c was only five-fold lower than that of polymerase α (137). Although in varying degrees, in all cell lines investigated to date, the replicase seems to be more sensitive to ara-c than is the repair DNA polymerase. Furthermore, this differential effect suggests a mechanism of action for ara-c which includes both the previously documented inhibition of DNA synthesis and the incorporation of this analog into DNA. One could speculate that high levels of ara-c are necessary to inhibit the repair enzyme which otherwise would excise the ara-c incorporated into DNA by the replicase.

A possible microscopic correlation of the molecular damage caused by the cell's inability to repair incorporated ara-c lesions is the appearance of chromosomal abnormalities. Ara-c can produce marked chromosomal aberration in mammalian cells with chromatid breaks and extensive fragmentations (5-7, 74, 81). Karon et al. (74) showed that ara-c cytotoxicity correlated very well with the number of chromosome breaks produced, five or more breaks per metaphase being lethal to the cell. The interference of ara-c with chromosomal integrity as a consequence of inhibition of DNA synthesis and repair would require that the cytotoxicity of ara-c be limited to cells rapidly incorporating DNA.

Ara-c is a cycle-dependent agent effective only in S-phase, which is the period of DNA synthesis in the cell cycle (55, 75, 82, 165). This S-phase specificity is due to the inhibitory effect of ara-c on DNA replication and to an increase in the phosphorylation of ara-c which results from an S-phase increase in deoxycytidine kinase activity (103). Preceding S-phase is G_1 phase during which the cells are spared the cytotoxic effects of ara-c.

Several investigators have noticed that ara-c can block the movement of G_1 cells into S-phase; thus, this drug can be self-limiting with respect to its cytotoxic activity (1, 55, 153). Although others have not observed a G_1/S block (76), the observation that ara-c induced partial cell synchronization strongly supports this effect (1, 8, 42, 85, 127, 159, 164). Consequently, cells collected near the G_1/S boundary could rapidly resume cell cycle traverse as a

synchronous cohort after ara-c is removed (1, 8, 42, 85, 127, 159, 164). Other implications of S-phase specificity will presently be discussed.

As it is with DNA, ara-c is also incorporated into RNA (29, 30, 33, 37). However, since ara-c causes only minimal impairment of general cellular RNA synthesis at doses that are inhibitory to DNA synthesis (36, 55, 108, 165), an effect on a specific RNA might be obscured. In fact, when RNA was fractionated, ara-c was not incorporated into high molecular weight rRNA (166) or tRNA (139) but was predominantly present in low molecular weight mRNA (30). Furthermore, Chu (30) has demonstrated that the cytotoxicity of ara-c correlates only with this analog's incorporation into low molecular weight mRNA. Even though mRNA is synthesized throughout the cell cycle (120, 155), and even though ara-c is S-phase specific, the cytotoxicity of ara-c could still result from an interaction with a specific S-phase mRNA. One type of S-phase specific protein is histones, which is a nuclear protein that is necessary for the assembly of the newly-formed DNA complexes (131). Histone synthesis requires a specific S-phase 7-9S mRNA(histone messenger) and Borun et al. demonstrated selective inhibition of histone messenger and histone synthesis by ara-c (12, 135). After treatment with actinomycin D and ara-c, both histone synthesis and the amount of 7-9S RNA (histone messenger) associated with polysomes declined four times faster than after exposure to actinomycin D alone. Since actinomycin D is known to block RNA transcription, ara-c's interference must occur after transcription to account for the rapid decay when synthesis of histone messenger is no longer occurring. Furthermore, one could

speculate that incorporation of ara-c into mRNA results in either accelerated metabolism of mRNA or in interference with translation.

The preceding argument strongly supports the theory that ara-c incorporation into mRNA is a mechanism of action of this drug. However, this hypothesis does not mitigate the importance of ara-c's inhibition of DNA polymerase or its incorporation into DNA. All of the aforementioned modes of action of ara-c could coexist, thereby explaining the potent cytotoxic nature of this compound.

There is yet another hypothesized locus of action for ara-c which, however, has been refuted by many investigators. Initial studies on the mechanism of ara-c cytotoxicity led to the suggestion that the drug produced inhibition of DNA synthesis as a consequence of the inhibition of the reduction of CDP to dCDP by the enzyme ribonucleotide reductase (31). However, when Skoog and Nordenskjold (142) measured nucleotide pools, ara-c caused only a transient decrease in the dCTP pool. In addition, when ribonucleotide reductase was assayed directly, ara-CTP produced a weak inhibition (73, 105), thereby suggesting that the inhibition of the reductase is not of significant consequence at the intracellular drug levels usually reached. Having discussed some of the proposed mechanisms of action of ara-c, it is important to consider the implications of these theories in the clinical application of the chemotherapeutic agent.

The clinical efficacy of ara-c as an antineoplastic agent is profoundly affected by the schedule of administration and dosage of this agent. After intravenous injection, ara-c is rapidly deaminated to

ara-u, uracil arabinoside (41). The plasma half-life for ara-c is biphasic with an initial fast phase (mean half-life 12 minutes) and a slower second phase (mean half-time 111 minutes) (65). In addition, Ho and Frei (65) demonstrated that the plasma half-life is independent of the dose; therefore, higher doses correlated with higher plasma levels of ara-c.

Since ara-c is S-phase specific and only a small percentage of tumor cells are in S-phase at a particular time, low dose continuous infusions have been commonly employed to expose a higher percentage of cells to ara-c during their vulnerable period (49). Yet, the efficacy of intermittent bolus therapy with appropriate dose intervals has also been demonstrated (110). The interval required for maximal cell kill correlates well with the time necessary for recovery of DNA synthesis following ara-c (110, 141). This observation suggests that ara-c synchronizes cells in early S-phase, and upon recovery these cells progress to another part of S-phase where they are sensitive to a second dose of ara-c (85, 110, 141). Recalling the previous discussion of the dose dependence of the mechanism of action of ara-c, it is therefore possible that large bolus doses could be acting at different sites and be as effective as prolonged low dose infusion.

If the cell kinetics of the tumor cells differ from that of the host tissues, use of an intermittent dose schedule could improve the therapeutic index of ara-c by decreasing host toxicity. For the treatment of acute leukemia, many clinical protocols employing intermittent doses or continuous infusions of ara-c have been tested.

Numerous protocols for the treatment of acute myelogenous leukemia (AML) have employed ara-c alone or in combination chemotherapy. Although these protocols differ widely in design, only the outcome of the most promising ones will be discussed as a standard of comparison for proposed new combinations. When used as a single agent for the treatment of adults with AML, ara-c has induced complete remission in approximately 25 percent of the patients (9, 54). A remission rate of 65 percent has been achieved by the combination of ara-c and thioguanine given every 12 hours until marrow hypoplasia resulted (34). In addition, the combination of a four-day course of daily ara-c with daunorubicin on day 1 induces complete remission in over 50 percent of patients with AML (110). A greater than 50 percent remission rate was also achieved with a complicated protocol involving ara-c, vincristine, prednisone and cyclophosphamide (150).

Ara-c has also been tried in the treatment of acute lymphocytic leukemia (ALL). However, since only one-third of patients with ALL achieved either a complete or partial remission with ara-c (144) and other agents are significantly more effective, the use of ara-c in ALL is limited to patients resistant to standard therapy.

L-Asparaginase

Since the original discovery of a tumor inhibitory factor in guinea pig serum by Kidd (64) and its identification as asparaginase by Broome (16), this enzyme has been shown to be tumoricidal to selected rodent (16, 80), canine (64, 113) and human (66) neoplasms.

Although asparaginase has been isolated from a wide variety of sources, the bacterial enzyme from E. coli is most often used clinically and for investigation (15, 116).

E. coli asparaginase (EC-2) is a 127,000 molecular weight protein that catalyzes the hydrolysis of asparagine to aspartic acid and ammonia (15, 17, 24, 37, 66, 95). In addition, as an inherent property of the enzyme, it has a small amount of glutaminase activity, which catalyzes the conversion of glutamine to glutamic acid and ammonia (116). Following the perenteral administration of asparaginase, plasma asparagine rapidly falls to undetectable levels (17, 24, 37, 66, 95, 130) and there is also a delayed transient decrease in glutamine (116).

The consequences of asparagine deprivation are the result of its important role in cellular function. The major metabolic use of asparagine is as one of the required amino acid constituents of proteins (24, 47, 95, 96, 147, 163). However, since most cells are capable of de novo biosynthesis of asparagine by the enzyme asparagine synthetase (16, 66, 122), asparagine is not an essential nutrient for mammalian cells (16, 17, 24, 116). In 1956, however, Neuman and McCoy (111) demonstrated for the first time a cell line, Walker Carcinosarcoma 256, which in contrast to normal mammalian cells has a nutritional requirement for asparagine. This finding has since been confirmed and extended to a limited group of tumor cells (15-17, 24, 58, 66, 92, 96). In fact, tumor cells that require exogenous asparagine as expected have either low or absent levels of asparagine

synthetase (17, 62, 119, 122). Furthermore, cells that require asparagine are susceptible to the lethal effects of asparaginase (17, 62, 66, 118, 119, 122) and the development of asparaginase resistance is associated with increased levels of asparagine synthetase (17, 62, 66, 118, 119, 122). Therefore, one can conclude that the antitumor action of asparaginase is the result of asparagine depletion (17, 62, 66, 118, 119, 122).

Although the mechanism of cell death following asparagine depletion has yet to be fully elucidated, the inhibition of protein synthesis following asparagine depletion or asparaginase has been well documented (24, 47, 89, 95, 96, 138, 147, 148, 163). One class of proteins that has a high asparagine content is glycoproteins (77). The high asparagine content of these proteins is very significant in that the oligosaccharide chains are covalently bonded to asparagine residues (24). Since cell membranes have a high glycoprotein turnover (14), and asparaginase inhibits glycoprotein synthesis (59, 71, 77), treatment with asparaginase could result in loss of membrane integrity (13, 14, 77, 78). Furthermore, such membrane damage would account for the observed rapid lysis of susceptible cells following asparaginase (43, 67). An alternate explanation of cell lysis has also been proposed by Dod et al. (43) who showed that asparaginase could directly solubilize partially purified cell membranes in vitro.

Aside from inhibition of glycoprotein synthesis, there are other important disruptings in cellular mechanisms induced by asparaginase.

Following the rapid inhibition of protein synthesis, there is a delayed inhibition of DNA (24, 47, 66, 89, 96, 148) and RNA (24, 47, 66, 89, 96, 148) synthesis. The delay suggests that the decrease in DNA and RNA synthesis is secondary to the inhibition of protein synthesis (47, 66, 96, 148).

The initiation of DNA synthesis requires the synthesis of special proteins (63, 121, 140). The inhibition of protein synthesis in late G_1 phase results in the inhibition of the initiation of DNA synthesis and hence blocks the transition of cells into S-phase (50, 68, 117). Asparaginase, as an inhibitor of protein synthesis, would thus be expected to block the initiation of DNA synthesis, resulting in an accumulation of cells at the G_1/S junction. In fact, an asparaginase-induced G_1/S block of cell cycle traverse has been reported by several investigators (86, 117, 136). However, a contradictory result was observed by Ernest (48), who demonstrated that asparaginase inhibited S-phase cells but did not block the G_1 to S transition. Finally, Paliardi et al. (115) were also unable to confirm an arrest of cell passage from G_1 to S-phase. Albeit that the effect of asparaginase on the cell cycle is controversial, most investigators feel that this effect is of secondary importance in contrast to the previously discussed independent lytic effect (48, 117).

Other hypotheses for the mechanism of action of asparaginase, which are independent of its effect on protein synthesis, have been described. Wood et al. (162, 163) have proposed that asparagine depletion, and therefore asparaginase, has a direct inhibitory effect

on DNA synthesis by blocking de novo biosynthesis of pyrimidines. They demonstrated that C^{14} asparagine is incorporated into pyrimidines, suggesting a role for asparagine as a precursor for pyrimidine synthesis. Even though asparagine is not directly involved in pyrimidine synthesis (88), its conversion to a required precursor such as glycine could explain the incorporation of label into pyrimidines. Meister (98) has described just such a pathway for glycine synthesis from asparagine. Moreover, if asparaginase inhibits glycine synthesis, the effects of asparaginase should be able to be blocked by the administration of exogenous glycine. In fact, not only can glycine antagonize the effect of asparaginase (130) but plasma levels of glycine fall following treatment with asparagine (129), further supporting the requirement of asparagine in glycine and nucleotide synthesis. However, significant decreases in intracellular pyrimidine pools following asparaginase have yet to be demonstrated and therefore, the importance of asparaginase inhibition of pyrimidine metabolism and DNA synthesis cannot now be determined.

Another site of action for asparaginase might involve the enzyme ribonuclease. An increase in ribonuclease, an enzyme that hydrolyzes RNA, has been proposed as a causal agent in the regression of murine lymphosarcoma P1798 (2) since only tumoricidal drugs including asparaginase caused this increase (2, 94, 97). Although this does not suggest a specific mechanism of action of this drug, it provides evidence that asparaginase may share a common final pathway with other agents in the induction of lymphocytolysis. Although 15 years have passed

since Broome (16) identified asparaginase as the cytotoxic component of Guinea Pig Serum, the exact tumoricidal mechanism of this enzyme is still unknown. However, this has not prevented the successful clinical application of asparaginase in the treatment of acute leukemia.

The clinical efficacy of asparaginase is almost entirely limited to the treatment of acute leukemia (24). Oettgen (112) has recently reviewed the clinical results of asparaginase therapy, alone and in combination with other drugs. The best responses to asparaginase therapy were in children and adults with acute lymphocytic leukemia. Of the 395 patients reviewed who were treated with asparaginase alone, 214 achieved either a complete or partial remission (54%) (112). However, of the 200 patients with acute nonlymphocytic leukemia, including acute myelogenous, acute myelomonocytic and acute undifferentiated, only 21% had either complete or partial remissions with asparaginase therapy alone (112).

Asparaginase in Combination Chemotherapy

Although the use of several combinations of asparaginase with other cytotoxic agents have been reported to be synergistic in animals and/or man, only the combinations of asparaginase with ara-c or methotrexate will be discussed here because of their direct importance to this thesis. Since ara-c and asparaginase are both useful drugs in the treatment of acute leukemia, and since they have different host toxicities (9, 54, 112), their combination is a logical choice. In fact, the combination of ara-c and asparaginase has been shown to

be more effective than either drug alone in the treatment of human and murine leukemias (4, 19, 46, 60, 87, 114, 124, 125). Although several trials have employed this combination in the therapy of acute lymphocytic leukemia (46, 60, 114, 125), there are no reports to my knowledge of such a trial in acute myelogenous leukemia.

The first clinical trial of the combination of ara-c and asparaginase was conducted by Hardisty and McElwain (60) who reported 8 complete remissions in 9 children with previously treated acute lymphocytic leukemia. The protocol consisted of using ara-c daily for 5-14 days followed by daily asparaginase for 9-28 days. The value of the sequential administration of ara-c, then asparaginase, was confirmed by Ekert et al. (46). They reported 81% complete or partial remission in 17 children with acute lymphocytic leukemia, using an ara-c bolus every 8 hours for 4 days followed by 4 daily doses of asparaginase. Finally, sequential administration of ara-c-asparaginase was also shown to be synergistic in murine leukemia EARAD (19). In contrast, simultaneous treatment with both ara-c and asparaginase has been reported to produce in acute lymphocytic leukemia remissions rates of 61% (15/24) and 68% (15/22) in two separate studies (114, 125). Simultaneous exposure in mice produced a synergistic response in the treatment of murine L5178Y when both drugs were administered in large doses on each of three separate days (4). Although all the reported protocols for the combination of ara-c-asparaginase resulted in an enhanced response rate as compared with asparaginase alone, the optimal schedule for the administration

of this combination has not yet been determined, but it is investigated further in this thesis.

For the treatment of murine and human leukemias, the importance of the schedule of administration of asparaginase with another S-phase specific DNA synthesis inhibitor, methotrexate, was first demonstrated by Capizzi et al. (22, 23, 25). They noticed that when mice with murine leukemia L5178Y were treated with asparaginase preceding methotrexate by less than 24 hours, there was an antagonistic response in mean animal survival time. However, when the order to the drugs was reversed and methotrexate preceded asparaginase, this antagonism was not observed. Furthermore, the subsequent treatment with asparaginase also decreased methotrexate's host toxicity and since the tumoricidal effect was not diminished, this regimen resulted in an enhanced therapeutic index. These results have been confirmed by other investigators (83, 156, 157).

Since ara-c and methotrexate are both S-phase specific inhibitors of DNA synthesis, their similarity suggested that their interaction with the protein synthesis inhibitor, asparaginase, might be similar. Therefore, the studies of Capizzi et al. (22, 23, 25) on the combination of methotrexate and asparaginase served as a basis for investigating whether the combination of ara-c-asparaginase also showed schedule-dependency. The purpose of this thesis is to determine the optimal schedule for the administration of ara-c and asparaginase and to attempt to clarify the biochemical basis of the interactions observed.

Methods

Leukemic Cell Lines

All animal and tissue culture studies were performed with the L5178Y/asn$^-$ murine leukemic cell line. These cells require asparagine for growth and therefore are sensitive to asparaginase (4, 58, 134). In addition, these cells are also sensitive to ara-c *in vivo* and *in vitro* (4, 32, 33).

Stock lines of L5178Y were maintained *in vivo* as an ascites tumor in 20-25 gram female AKD_2F_1 mice (Jackson Laboratories, Bar Harbor, Me.). The tumor was transferred at weekly intervals into new mice by intraperitoneal injection of 10^6 cells. For *in vitro* studies, stock lines of L5178Y were maintained as liquid suspension culture in Fisher's medium supplemented with 10% horse serum (designated FS_{10} and purchased from Grand Island Biological, New York) and were kept in continuous logarithmic growth by frequent subculturing. Fisher's medium was protected from light by wrapping the bottles in aluminum foil in order to prevent the formation of toxic photoxidation products (3, 150). All cultures were maintained at 37° C, in a 5% CO_2 incubator and under these conditions, these cells had a mean doubling time of 8.5 hours.

In Vivo Tumor Growth Curves

Mice were inoculated intraperitoneally with 10^6 L5178Y ascites

tumor cells in a volume of 0.1 ml diluted in sterile normal saline (NSS). On day 3, animals were treated with either ara-c 1000 mg/kg (Upjohn, Kalamazoo, Mich.), asparaginase 500 IU/kg (Merck, Sharp and Dome, Pa.) or normal saline. The drugs were freshly prepared from sterile powder in normal saline and the final injection volume was 0.1 ml. At various intervals, two animals were sacrificed by cervical dislocation. The skin over the peritoneum was opened and the peritoneal cavity was lavaged with three 5 ml aliquots of heparinized normal saline (0.5 IU/ml) to collect the tumor cells. After dilution with NSS and the addition of 3 drops of Zapisoton (Coulter Electronics, Hialeah, Fla.) to lyse the red cells, the tumor cells were counted in a Model A Coulter counter (Coulter Electronics, Hialeah, Fla. This counter was used for all experiments in this thesis). Results were expressed as total cells per animal.

Animal Survival Studies

Six groups of 6 mice were segregated into separate cages and inoculated with tumor and treated with drugs that were prepared as described above. The treatment schedule is described in Table 1. As an indication of drug toxicity, each group of animals was weighed daily until they regained their pretreatment weight and there were no deaths due to drug toxicity in any of these studies. Results were expressed as mean animal survival in days following tumor injection and 60 day survivors were considered cured.

In Vitro Cell Viability

Thirty-five ml of L5178Y cells in log phase growth at a concentration of 1-3 X 10^5 cells/ml were treated with 1 ml of a drug freshly prepared in NSS according to the treatment schedule in Table 2. Pre- and post-treatment cell counts were determined in a Model A Coulter Counter after a 1:10 dilution with NSS. Following an 8-hour drug exposure, the cells were washed three times by centrifuging them for 10 minutes at 1000 rpm in a Sorvall GLC-2 centrifuge (Dupont Industries, Newtown, Conn.) in a 37° room and then resuspending the cells in 35 ml of drug-free FS_{10} by gentle pipetting. One hour and thirty minutes after concluding the first centrifugation, the cells were treated a second time as diagrammed in Table 2. At the conclusion of a second 8-hour exposure, the cells were again counted and washed as above and viability was determined by a slight modification of the soft agar cloning technique described by Fisher and Chu (32). In contrast to the 60 cells/tube used by Fisher and Chu, the inoculum used in these experiments was either 100 and/or 200 cells per tube depending upon the anticipated cell kill. Cloning efficiency varied, the mean being 75% and the data was therefore normalized and expressed as percent of control cloning efficiency.

A sequence of experiments was undertaken to determine if the 1 hour and 30 minutes between treatments was critical. The four time intervals tested were as follows: a) 1 hour overlap b) 1 hour thirty minutes c) 4 hours d) 8 hours. In group A the second drug was added 1 hour prior to the conclusion of the first treatment and

was therefore included in the FS_{10} used in washing. The treatment schedule is diagrammed in Table 3.

In Vitro Growth Curve Following Ara-c Pretreatment

L5178Y cells in culture were treated with either ara-c (final concentration 10^{-6} M) or normal saline for 8½ hours as described above for the first treatment of the *in vitro* viability studies. Following the resuspension of the cells after the third wash, hourly cell counts in duplicate were measured by diluting 1 ml of cells with 9 ml of NSS. Prior to counting, the diluted cell suspensions were dispersed with a pasteur pipette. A graph of cell concentration as a function of time post-wash was used to express these results.

Incorporation of TdR into DNA

L5178Y cells in culture were pretreated as described above for the *in vitro* growth curve. Following the third wash the cells were resuspended in FS_{10} to a final concentration of 3-6 X 10^5 cells/ml. One ml of cell suspension was then placed in a sterile, stopperred, 10 ml Erlenmeyer flask that already contained either 0.1 ml of normal saline solution or 0.1 ml of asparaginase (6 IU/ml prepared in NSS). Following a 1 hour preincubation at 37° C in a Metabolyte shaking water bath (New Brunswick Scientific Company, New Brunswick, N.J.), 0.1 ml of H^3 deoxythymidine (1 uc of H^3 methyl TdR diluted in Fisher's medium. Specific activity 2.0 C/mM from New England Nuclear, Boston, Ma.) was added to the incubation mixture. At 10 minute intervals

for the next 1 hour, 0.1 ml of the mixture was removed and applied to a glass fiber disc (2.4 cm GF/A Whatman filter) which had been pre-treated with 0.2 ml of 0.5 M NaOH and allowed to dry. At the conclusion of the incubation, the discs were dried under a heat lamp and placed on Whatman #1 filter paper in an 18 cm Buchner funnel mounted on a suction flask. Up to three layers of discs, separated by Whatman #1 filters were washed with ten 100 ml aliquots of ice cold 5% trichloroacetic acid (TCA) followed by four 100 ml aliquots of 95% ethanol and then four 100 ml aliquots of acetone. The discs were again dried and then transferred to scintillation vials with 10 ml of Econofluor (New England Nuclear, Boston, Ma.). The samples were counted in a Packard Tricarb Liquid Scintillation Counter (Packard Electronics). The rate of incorporation of H^3 TdR into DNA was calculated using linear regression analysis and the results were expressed as dpm/10^6 cells/minute of incubation.

An alteration of this general method was used when it was desirable to determine DNA synthesis within 1 hour post-ara-c treatment. For these experiments, the cells were divided into two groups, half was washed with FS_{10} plus asparaginase (0.5 IU/ml) and the other group without asparaginase. Also to accomplish the removal of the drug within 1 hour, the washing was reduced to three 7 minute centrifugations. At 1 hour 1 ml of cells was placed in a 10 ml flask and 0.1 ml of H^3 TdR was immediately added since the 1 hour wash with asparaginase was approximately equivalent to preincubating with the drug for 1 hour.

H^3 Ara-c Incorporation into Macromolecules

Two ml of 1-2 X 10^6 L5178Y cells in FS_{10} were added to a 10 ml sterile, stopperred Erlenmeyer flask that contained 0.2 ml of H^3 ara-c (5 uc of specific activity 13.2 mc/mM. Prepared in Fisher's medium and purchased from New England Nuclear, Boston, Ma.). In order to determine if asparaginase inhibited ara-c's incorporation into DNA and/or RNA, six flasks of cells were treated as follows: two of the flasks received 0.1 ml asparaginase (12 IU/ml) initially, two others received 0.1 ml of asparaginase (12 IU/ml) after 2 hours and the remaining two flasks received 0.1 ml of normal saline. Every thirty minutes for 4 hours, 0.2 ml of the mixture was removed and spotted on glass fiber discs that were previously treated with both 0.2 ml of 0.5 M NaOH and 0.2 ml 10^{-4} M cold ara-c. The discs were then washed and counted as described above for TdR.

H^3 Leucine Incorporation into Protein

Cells were prepared as previously described for TdR but received 0.1 ml H^3 Leucine (2 uC of 4,5H^3 leucine specific activity 5 C/mM purchased from New England Nuclear, Boston, Ma.). The 0.1 ml aliquots were pipetted into 3 ml of ice cold 10% Trichloroacetic acid and the samples were processed and counted by the method described by Rosenfelt (128).

Autoradiography and Mitotic Index

One ml of 2 X 10^6 L5178Y cells was placed in a stopperred, sterile, 10 ml Erlenmeyer flask with 0.1 ml H^3 UdR (1 uc of 25 C/mM 5,H^3 from

New England Nuclear, Boston, Ma.) and 0.1 ml colcemid (final concentration 0.05 ug/ml from Grand Island Biological, New York). At the conclusion of a 1 hour incubation, the cells were washed two separate times with 3 ml of cold normal saline and then resuspended in 2 drops of horse serum. After the slides were prepared and dried, they were fixed for 15 minutes in absolute methanol. The slides were developed by the method of Durie and Solomon (18) with the exception that after dipping them in the emulsion they were not redipped into scintillation fluid and consequently exposure time was increased to 4 days. The autoradiograms were stained with Geimsa and Wright stain and 500 cells on duplicated slides were counted to determine the mitotic and labeling index.

Results and Discussion

The design of a chemotherapeutic protocol for the use of asparaginase plus ara-c should be guided by the pharmacology of the individual agents as reviewed above. Although it is impossible to predict *a priori* the interactions that will occur, an initial hypothesis can be based on known cytokinetic effects. Since ara-c is an S-phase specific agent (55, 75, 82, 165) which is therefore most effective against rapidly dividing cells, an additive or synergistic response might be expected to occur if cells were treated with ara-c shortly after they were released from the inhibition of asparaginase which has been reported to cause a G_1/S block (86, 117, 133). To explore this hypothesis, a preliminary group of experiments was necessary to determine when L5178Y cells recover from asparaginase toxicity *in vivo*. Therefore, a tumor growth curve in mice following asparaginase was determined as described above. The pooled data of two experiments is presented in Figure 1. After a single injection of asparaginase, there is a greater than one log cell kill followed by a plateau of cell number for 4 days. Between 120 and 144 hours post-treatment, the remaining tumor cells resume logarithmic growth. A similar observation has been previously reported by Rosenfelt (128). Consequently, one of the treatment regimens tested in mice was the administration of asparaginase on day 3 post-inoculation with tumor and

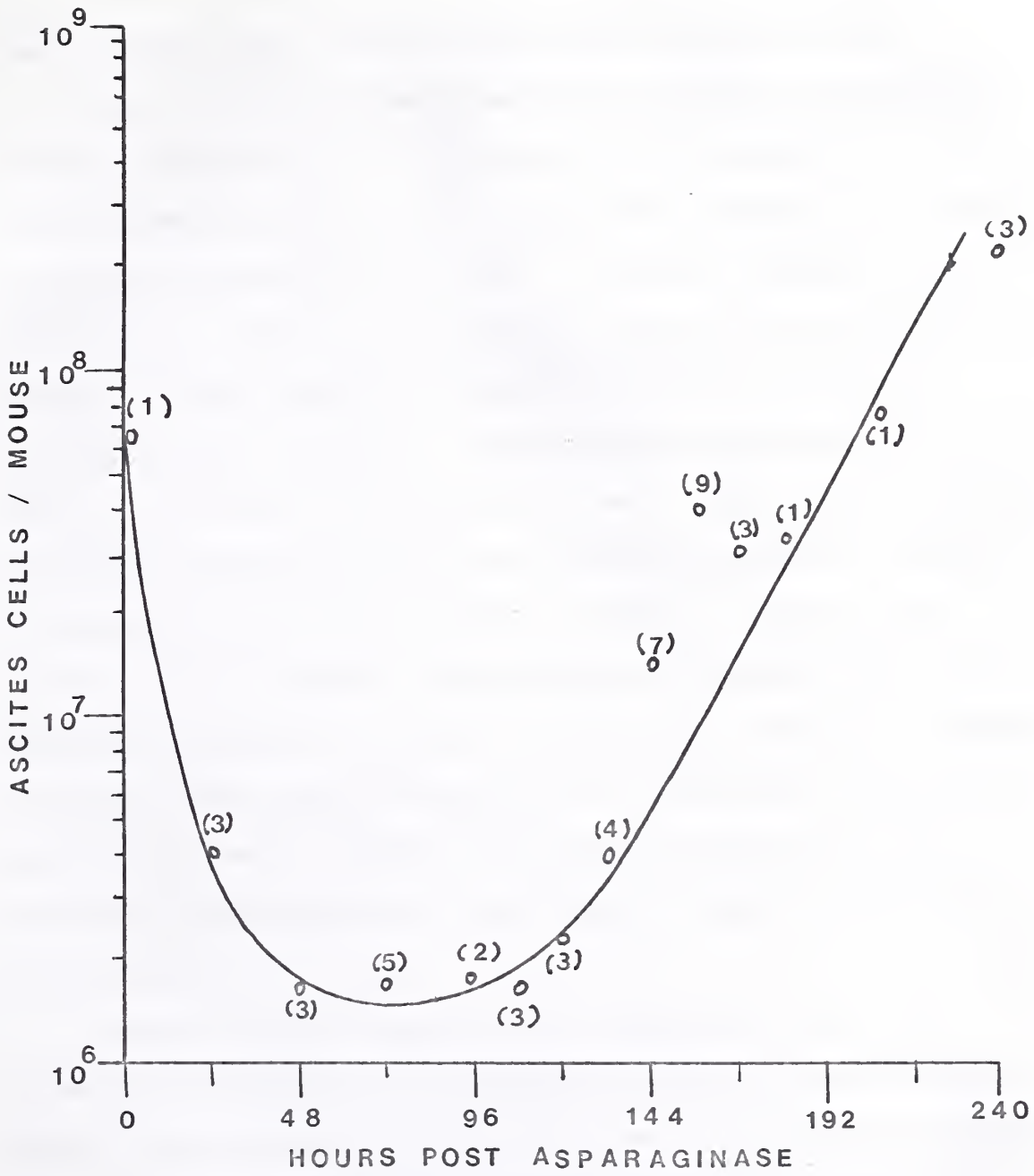


Figure 1:Effects of 500 IU/kg of Asparaginase upon the total number of ascites cells per mouse. Data points represent the pooled results of two experiments. ( )=number of animals per time point and the point is the mean of those animals.

then 120 hours later a single bolus of ara-c was administered.

Another initial hypothesis was based on a presumed asparaginase-induced G_1/S block (86, 117, 133) which has been suggested to result from the inability of the cell to initiate DNA synthesis in the absence of protein synthesis (63, 121, 140). In addition, recovery from the DNA synthesis inhibitor, ara-c, probably requires de novo synthesis of certain proteins. Therefore, it might be optimal to treat cells with asparaginase just prior to their resumption of DNA synthesis while in the process of recovering from ara-c. Under these conditions, asparaginase might block cells from recovering from ara-c's toxicity. To determine the appropriate treatment interval, an in vivo tumor growth curve following ara-c was determined as described above and is presented in Figure 2. This growth curve implies that the cell number increases rapidly after 144 hours and it is thus likely that most remaining cells begin to recover approximately 120 hours after ara-c. Therefore, in the design of a trial treatment schedule, asparaginase followed ara-c by 120 hours.

Finally, the third schedule of the combination of ara-c and asparaginase tested was the simultaneous treatment with both drugs on day 3. This was selected because it has been reported to be clinically useful (114, 125) and also to serve as a control for the other protocols.

The treatment schedules and results of the animal survival studies are presented in Table 1. Treatment with ara-c alone prolonged the life of all mice and cured 3 of 24. Furthermore, treatment with

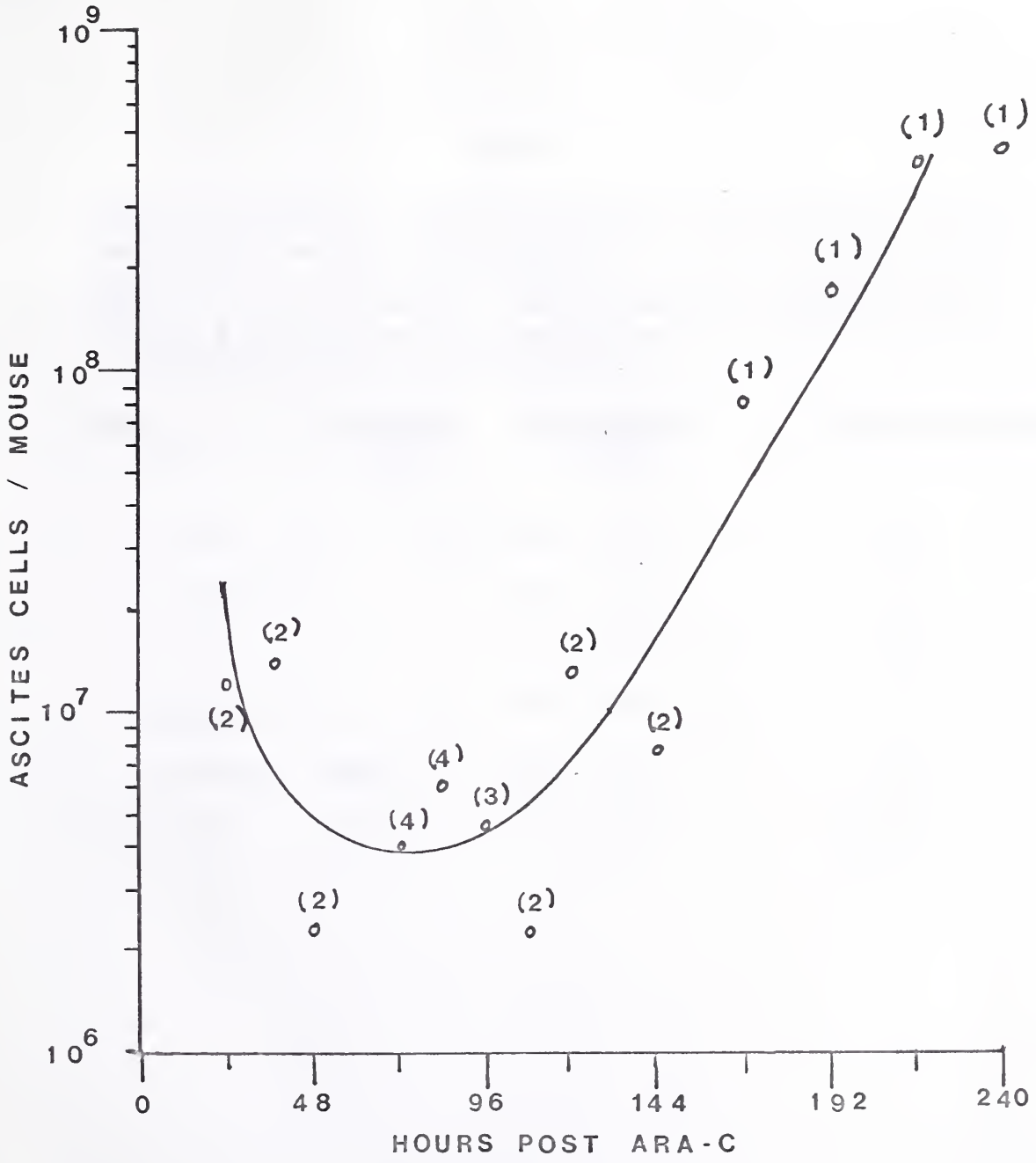


Figure 2: Effect of 1000 mg/kg of ara-c upon the total number of ascites cells per mouse. Data points represent the results of one experiments. ( )=number of animals per time point and the point is the mean of those animals.

Table I

Effect of different treatment schedules on mean survival time and the number of animals cured following IP injection of 10^6 tumor cells on day 0. All drugs delivered in 0.1 ml. Asparaginase= 500 IU/kg. Ara-c = 1000 mg/kg. The results are expressed as Mean Survival ± standard deviation and represent the pooled data from 4 experiments. Cure= 60 day survivors.

Group	Treatment Day 3	Treatment Day 8	Mean Survival (Days)	Cured/Total Animals
I	Saline		10.1 ± 1.7	0/46
II	Asn'ase		16.4 ± 1.8	0/34
III	Ara-C		21.0 ± 2.7	3/24
IV	Asn'ase + Ara-c		18.1 ± 1.8	1/24
V	Asn'ase	Ara-C	21.9 ± 4.3	1/24
VI	Ara-C	Asn'ase	30.3 ± 0.9	20/24

asparaginase alone cured none of the animals and although it prolonged the life of all animals, asparaginase was slightly less effective than ara-c alone. When animals were treated simultaneously with both drugs or when asparaginase preceded ara-c by 120 hours, animal survival was equivalent to ara-c alone. However, when ara-c preceded asparaginase by 120 hours, there was a marked synergistic response with 83% (20/24) of the animals being cured. In addition, there were no toxic deaths and the maximum weight loss in any group was 10%. These experiments demonstrate a schedule-dependent synergistic protocol for the treatment of mice carrying L5178Y leukemia and this protocol has minimal host toxicity as measured by weight loss.

The effect of the combination of ara-c and asparaginase on animal tumor models has been reported by other investigators (4, 19). In contrast to the results of this study, Avery and Roberts observed that the simultaneous treatment of L5178Y bearing mice with these two drugs on each of three days (day 1, 4, and 7) cured 36 of 43 mice (146). Furthermore, three doses of ara-c alone cured none of the animals, and three doses of asparaginase cured only 3 of 15 mice. Even though the drug doses used by Avery and Roberts for each injection were similar to those employed in this study, their use of multiple doses and short intervals between doses complicates the comparison of these two experiments. In addition, based on the studies of Avery and Roberts it is difficult to separate the interaction of the simultaneous administration of both agents from the effects of either drug with a subsequent dose. In fact, the synergism observed by Avery and Roberts may actually support the advantage of the sequential

administration of ara-c and then asparaginase if as a consequence of multiple doses, asparaginase is enhancing the effect of a previous dose of ara-c. The superiority of the sequential regimen is further supported by an animal survival equivalent to that reported by Avery and Roberts for the studies reported here despite a larger tumor burden at the time of initiating therapy. The animals used here had a larger tumor burden since treatment was initiated on day 3 in contrast to the mice used by Avery and Roberts which began receiving therapy on day 1. The delay of therapy to day 3 results in the mice having at least one log more cells than on day 1, thereby increasing the difficulty of curing the animals (158).

A synergistic increase in animal survival for the combined use of ara-c and asparaginase in treating murine leukemia $EARAD_1$ has also been reported by Burchenal (19). In that study, 7 of 10 mice were cured with ara-c 10 mg/kg on days 3-7 followed by asparaginase 500 IU/kg on day 7. In addition, a direct comparison between the studies of Berchenal and those reported here is complicated by different animal tumor model, dosage schedules for ara-c, and intervals betewen ara-c and asparaginase. However, analysis of these two studies raises the possibility that the 120 hours interval between ara-c and asparaginase used in this project may not be critical and that further studies are needed to define the precise schedule dependency of this combination.

In view of the efficacy of the sequential administration of ara-c, then asparaginase <u>in vivo</u> as described above, experiments were conducted in cell culture to define the nature of the drug-drug

interaction devoid of host-mediated effects. Prior to investigating the effects of the combination on cell viability, it was necessary, as before, to determine when the cells begin to recover from ara-c toxicity. Therefore, an in vitro growth curve as described under Methods was done. A typical growth curve is presented in Figure 3 and it shows that following a 2-hour lag, the ara-c pretreated cells begin to rapidly increase in number. In fact, the shorter doubling time for the midpoint of the ara-c curve (6.8 hours) as compared to the control (11.0), suggests that ara-c pretreatment may have partially synchronized the cells. The synchronization with ara-c will be discussed in detail below.

Data derived from the in vitro growth curve implied that cells resume active growth 2 hours after ara-c's removal. Therefore, if asparaginase treatment was to precede the recovery of cells from ara-c, the addition of asparaginase would have to be within 2 hours of drug removal. Since 90 minutes is the minimum time for washing a large number of samples, this was selected as the time interval between treatments in the cell viability studies. The treatment schedule and results of these experiments are presented in Table 2. At a concentration of 10^{-6} M, ara-c was moderately toxic with a mean viability of 52.8%. The dose of asparaginase chosen (0.5 IU/ml), however, was more lethal and the mean viability was only 23%. The evaluation of the combination requires an understanding of the anticipated response. For in vitro cell viability studies, an additive response is defined as the product of the percent viability of the two single drugs and

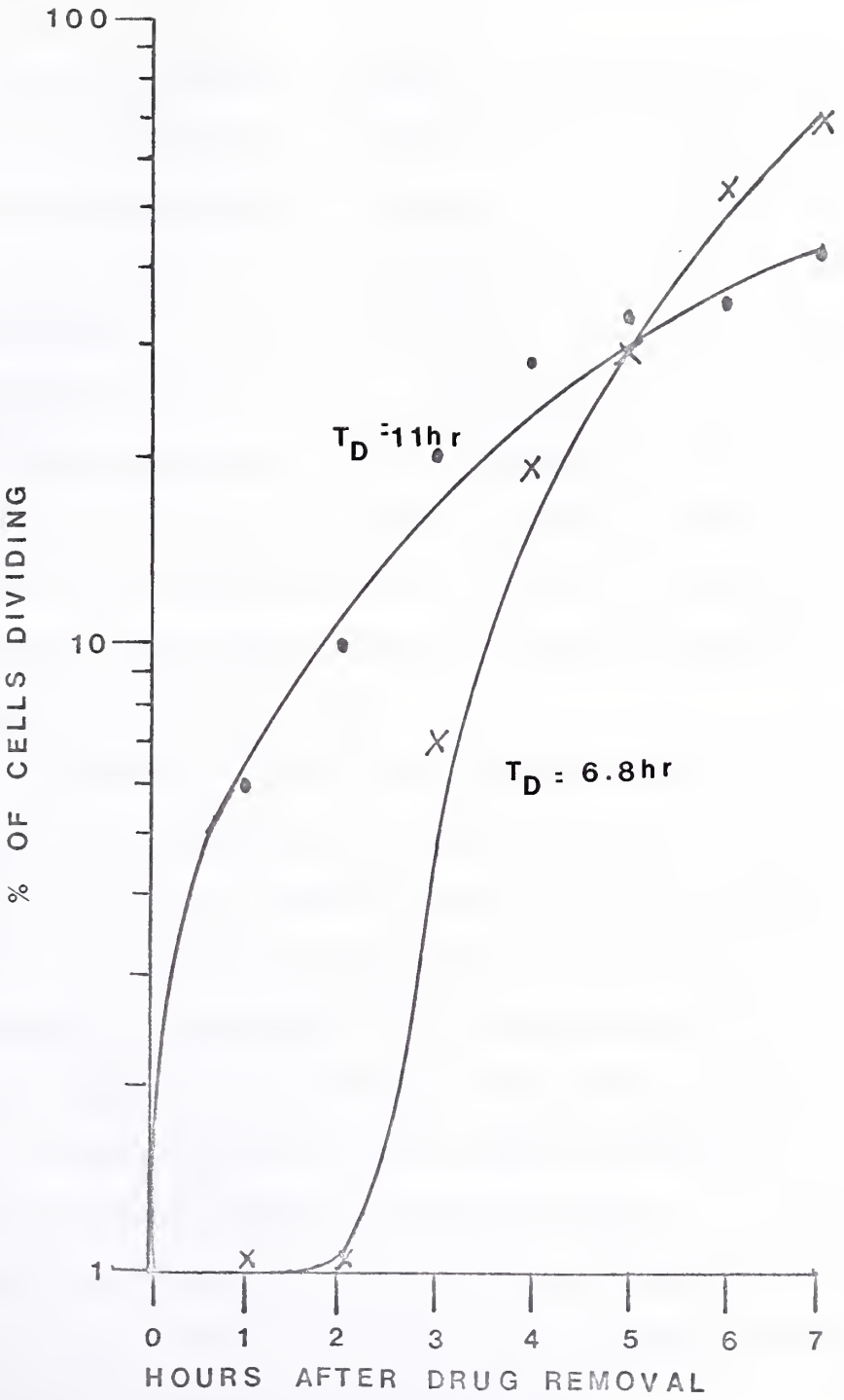


Figure 3: Outgrowth of L5178Y cells in culture following 8 hours of treatment with either normal saline(●) or ara-c (X).

a response with greater cell kill is considered synergistic (158). Using this definition, an additive response for ara-c plus asparaginase would be expected to result in a 13% viability. The 7% viability observed for the sequential treatment with ara-c and then asparaginase is at least additive if not synergistic. However, the simultaneous addition of both drugs (22.8%) was no more effective than asparaginase alone (23.0%). Furthermore, when asparaginase preceded ara-c (50.3%) an antagonistic response occurred with the combination being significantly less effective than asparaginase alone ($p > .005$). These results support the schedule-dependent synergy and antagonism observed in vivo.

To determine if the 90 minute interval between treatments was critical, two experiments were performed using the protocol outlined in Table III and the normalized results are also presented in Table IV. Figure 4 is a graphic representation of the means of both experiments expressed as a percentage of the anticipated additive response for both drugs versus the time post-wash. This graph shows that the synergistic response observed when ara-c precedes asparaginase is lost when the interval between treatments exceeds 90 minutes. In conjunction with the data from the growth curve, this result implies that for asparaginase to be effective, the cells must be treated prior to their resumption of active growth following ara-c.

The curve for treatment group 5 (asparaginase preceding ara-c) in Figure 4 suggests that the longer the interval between the two drugs the greater the degree of antagonism. At first analysis, it

Table II

Effect of different treatment schedules on cell viability *in vitro*. Ara-c=10^{-6}M. Asn'ase=asparaginase 0.5 IU/ml. Viability expressed as a percentage of control ± one standard deviation.

Protocol	Treatment 1 (8 hours)	Wash (90 min.)	Treatment 2 (8 hours)	Wash	Viability	No. Exp.
I	Saline		Saline		100	
II	Saline		Ara-C		52.8 ± 19.3	10
III	Saline		Asn'ase		23.0 ± 11.1	10
IV	Ara-C		Asn'ase		7.0 ± 4.0	4
V	Asn'ase		Ara-c		50.3 ± 17.4	4
VI	Saline		Ara-c plus Asn'ase		22.8 ± 5.7	9

Statistical Significance (Student's T test)

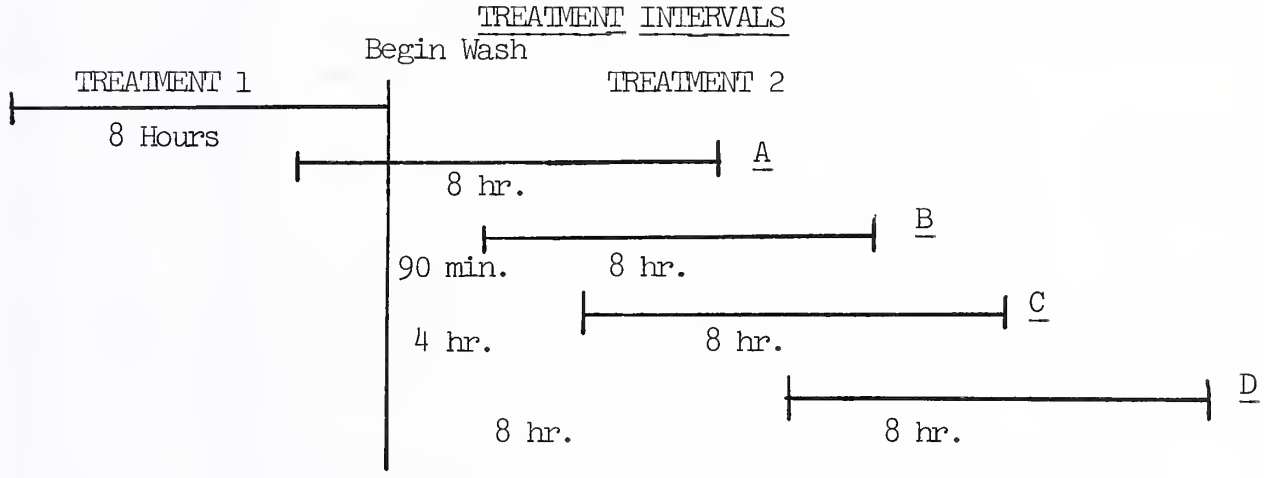
II	vs	IV	P .001
II	vs	V	NS
II	vs	VI	P .005
III	vs	IV	P .025
III	vs	V	P .005
III	vs	VI	NS
V	vs	IV	P .001

NS=not significant

Table III

The Effects of the Treatment Interval on Cell Viability in Vitro

Group	Treatment 1	Treatment 2
I	Saline	Saline
II	Saline	Ara-c
III	Saline	Asparaginase
IV	Ara-c	Asparaginase
V	Asparaginase	Ara-c
VI	Saline	Ara-c plus Asparaginase



Interval	Group	Viability (% of Control) EXP 1	EXP 2
A - 1 hr. overlap	I	100	100
	II	72	87
	III	30	44
	IV	12	10
	V	71	68
	VI	28	24
B - 90 min.	I	100	100
	II	54	64
	III	30	22
	IV	13	5
	V	53	72
	VI	30	14
C - 4 hours	I	100	100
	II	56	46
	III	33	10
	IV	36	18
	V	50	42
	VI	24	15
D - 8 hours	I	100	100
	II	24	58
	III	17	10
	IV	17	14
	V	38	57
	VI	23	28

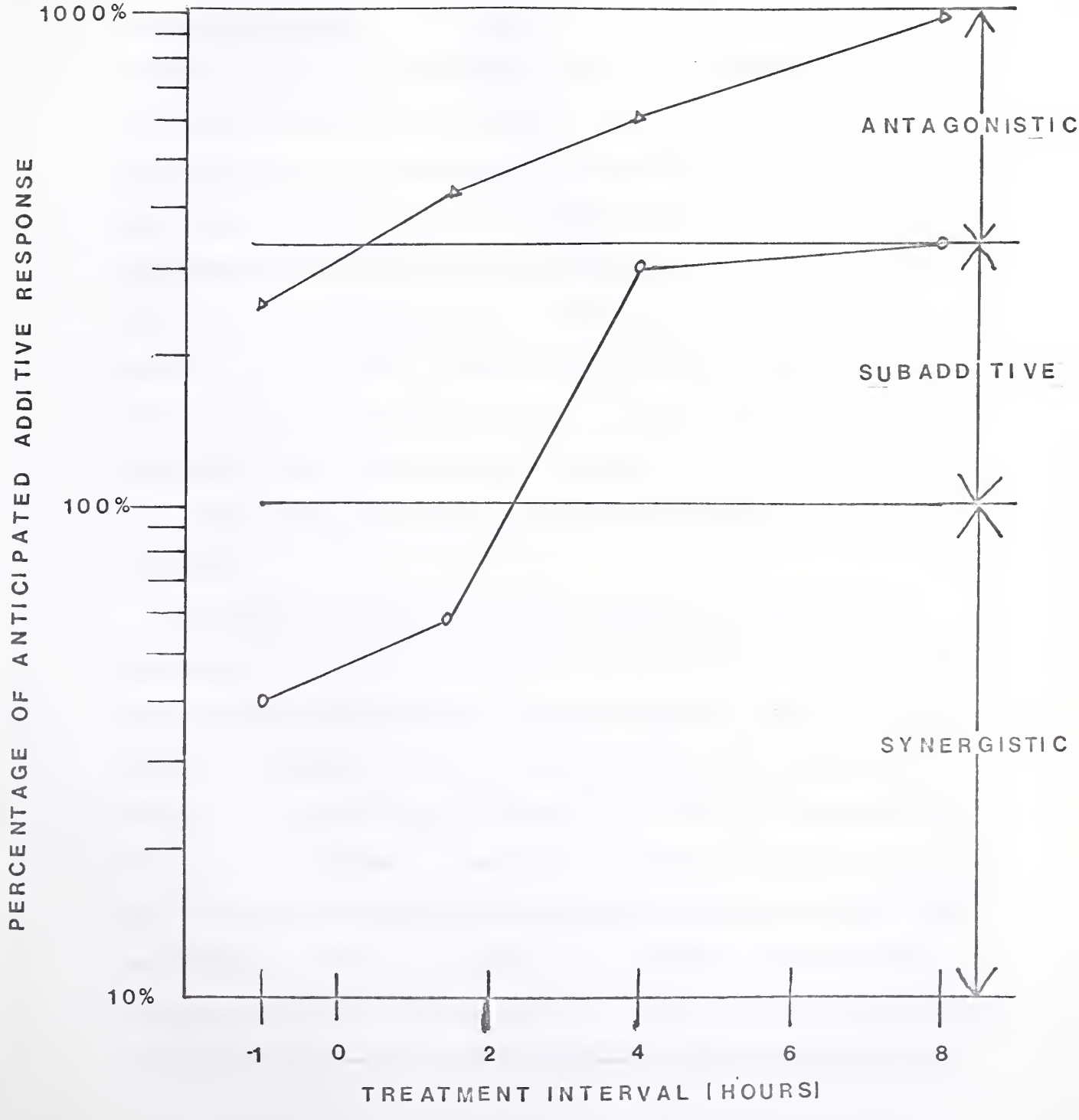


Figure 4: Effect of the treatment interval on cell viability as a percentage of the expected response (as explained in text) following treatment with both ara-c and asparaginase. The line at 330% is equal to the effect of asparaginase alone as a percentage of the anticipated additive response. Data points=average of two experiments.
▲ ASN'ase then ara-C
○ ara-C then asn'ase

appears that ara-c is actually rescuing cells from asparaginase toxicity. However, an alternate explanation can be derived from the pre- and post-wash cell counts. In contrast to ara-c, when cells are washed following this dose of asparaginase, there is a mean cell loss of 75% as compared with the pretreatment cell number. This implies that the cytotoxicity of asparaginase is underestimated by the cell viability since there is a large degree of lymphocytolysis. Cell lysis following asparaginase has been previously reported (136, 139). The longer the interval post-asparaginase the greater the number of cells that can express the lytic affect of asparaginase toxicity. Therefore, since cloning only represents the viability of intact cells, the apparent antagonism of this combination at treatment intervals of 4 or 8 hours may be only artifactual because asparaginase's toxicity is underestimated.

The data presented above strongly supports the superiority of the sequential administration of ara-c and asparaginase. One possible explanation for the efficacy of this drug regimen is that ara-c pretreatment potentiates the effect of asparaginase on macromolecular synthesis. To explore this hypothesis, the effect of asparaginase on the rate of DNA synthesis as measured by TdR incorporation in cell culture following ara-c treatment was determined as described above and the results are summarized in Table IV. Contrary to the above hypothesis, both the ara-c pretreated and the control group had approximately a 60% inhibition of DNA synthesis following 1 hour preincubation with

Table IV

Effect of Asparaginase on H^3TdR incorporation 210 minutes after beginning washing following 8 hour pretreatment with either saline or ara-c 10^{-6}M. Data represents the mean and standard deviation of 6 experiments. Rate=dpm/10^6 cells/minute incubation.

Protocol	Rate	%Control	% Inhibition
Control	15220 ± 3618		
Control + Asparaginase	5925 ± 893		61.1%
Ara-c	21390 ± 4685	141%	
Ara-c + Asparaginase	9075 ± 1153		57.8%

Table V

Effect of Asparaginase on H^3TdR incorporation 210 minutes after beginning washing following 8 hour pretreatment with either saline, ara-c 5 X 10^{-6}M or ara-c 5 X 10^{-7}M. Data represents the mean of two experiments. Asparaginase=0.5 IU/ml as described in text. Rate=dpm/10^6 cells/minute of incubation. Control is the same as in Table IV.

Protocol	Rate	% Control	% Inhibition
Control	15220		
Control + Asparaginase	5925		61.1%
Ara-C (5 X 10^{-6}M)	8888	41.6%	
Ara-c (5 X 10^{-6}M + Asparaginase)	4375		50.8%
Ara-c (5 X 10^{-7}M.)	16353	107%	
Ara-c (5 X 10^{-7}M. + Asparaginase)	8062		50.7%

asparaginase. Furthermore, in order to exclude the possibility that the degree of inhibition with asparaginase might be a function of the concentration of ara-c used, the results of three different dosages (5×10^{-6}, 1×10^{-6} and 5×10^{-7}) were determined and are summarized in Table V and the variation in inhibition ranging from 50 to 58 percent is probably not significant.

Finally, studies were undertaken to determine if the percentage inhibition of DNA synthesis induced by asparaginase was constant at different times post-wash. These results are presented in Table VI and Figures 5, 6. As expected, Figure 5 demonstrates a constant rate of DNA synthesis for the control. However, following ara-c's removal DNA synthesis is initially inhibited but at 210 minutes, it reaches a peak of 141% of control. This curve suggests S-phase synchronization and will be discussed in detail below. Figure 6 shows that although both the rate of DNA synthesis and the percentage inhibition with asparaginase is constant for the control, following ara-c pretreatment, there is variation in both parameters. Even though this graph represents the average of only two experiments, the general configuration of the curve suggests that the higher the rate of DNA synthesis, the greater the inhibition induced by asparaginase. The significance of this finding is difficult to determine in light of two contradictory lines of evidence. First, although the rate of DNA synthesis 210 minutes post-wash in the ara-c pretreated group is 141% of control (Figure 5), the percentage inhibition with asparaginase in both groups is 63% (Table VI). Second, since the inhibition with

Table VI

Effect of the post wash interval on TdR incorporation with and without asparaginase following either saline or 10^{-6}M ara-c for 8 hours. Asn'ase=asparaginase 0.5 IU/ml. % Control= rate of ara-c divided by the rate of control. Results are the average of two experiments. Rate=dpm/10^6cells/minute incubation.

Time	Group	Rate	Rate with Asn'ase	%Inhibition	% Control
90 min.	Control	17128	6993	59.2%	
	Ara-C	8820	4908	44.4%	51.5%
210 min.	Control	18067	6540	63.8%	
	Ara-C	26763	10086	62.3%	148%
6 hours	Control	18067	6110	66.2%	
	Ara-C	14632	7752	47.0%	80.1%
8 hours	Control	17168	5905	65.6%	
	Ara-C	20662	6445	70.1%	120%
10 hours	Control	19377	7645	60.5%	
	Ara-C	19955	8395	57.9%	103%

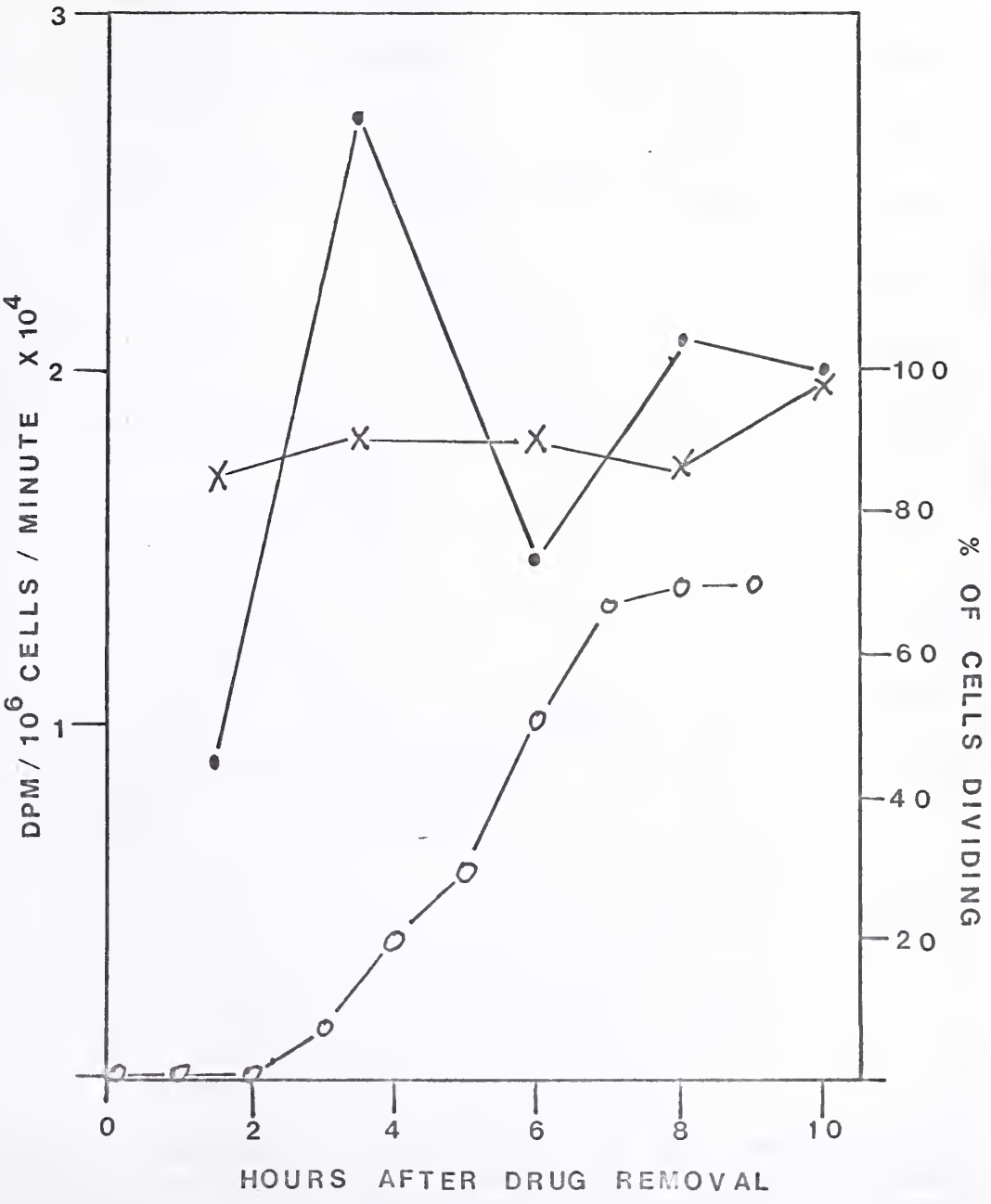


Figure 5: Effect of the post wash interval following ara-c on the rate of TdR incorporation into DNA. Each point is the average of two experiments. (X)=normal saline (●)=ara-c 0---0 =Outgrowth curve for cell number following ara-c see figure 3.

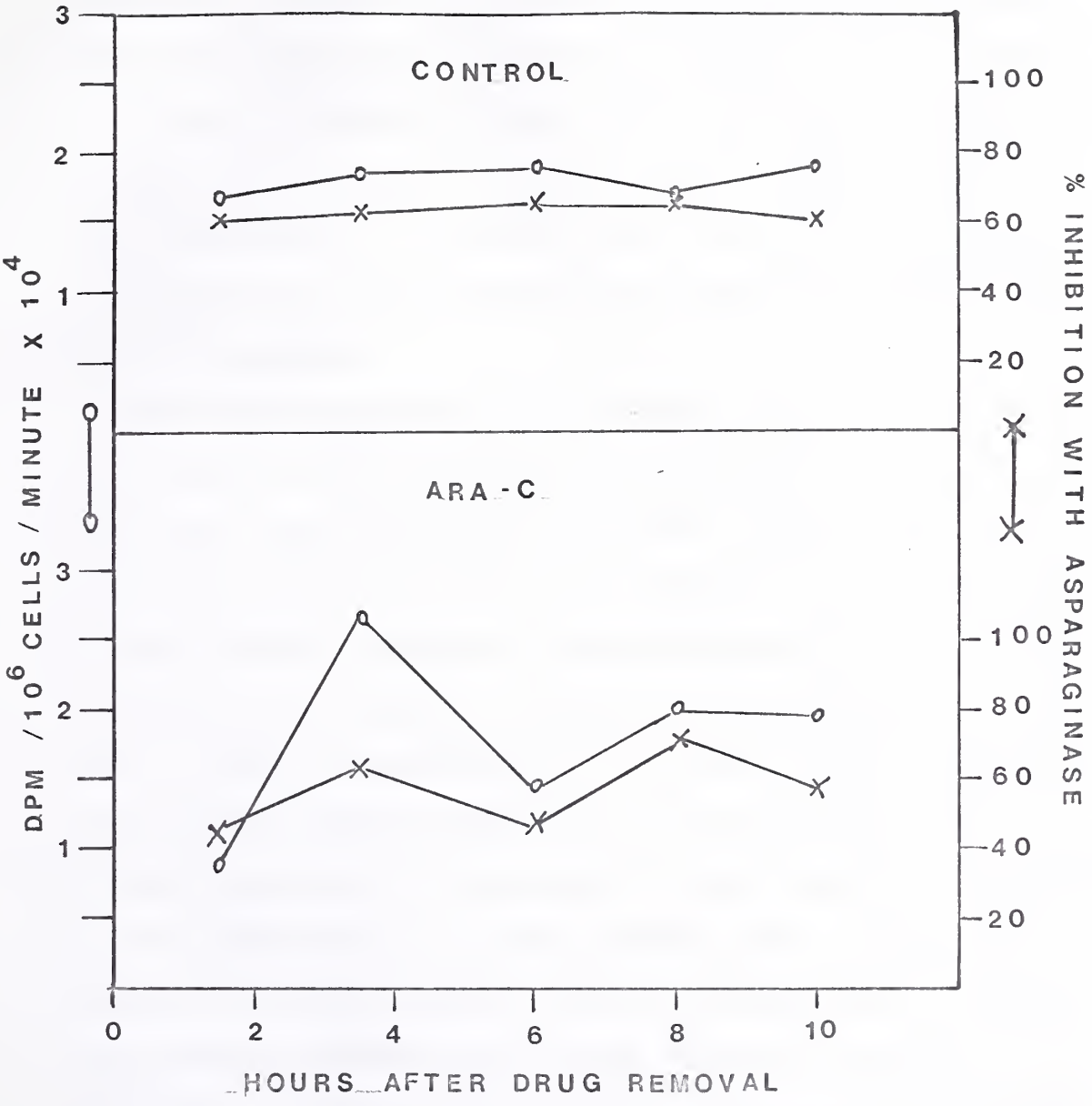


Figure 6: Exponetially growing L5178Y cells were treated with ara-c for 8 hours and then the rate of DNA synthesis and the inhibitory effect of 0.5 IU /ml of asparaginase on DNA synthesis was determined as a function of time after removal of ara-c. (0)=Rate of DNA synthesis (dpm/10^6 cells/minute incubation) . (X)=% inhibition with asparaginase.

asparaginase is greater at 210 minutes in the ara-c pretreated group than at 90 minutes, one would expect asparaginase to induce a greater cell kill at 210 minutes. In fact, the viability studies previously discussed showed equivalent cytotoxicity for both time intervals (Table III, Figure 4). In summary, these experiments do not support the hypothesis that ara-c potentiates the asparaginase-induced inhibition of DNA synthesis.

An alternate hypothesis for the synergy of this combination can be proposed based on the observation in Figure 5. The higher than control rate of DNA synthesis post ara-c (141% of control at 210 minutes) is consistent with partial S-phase cell synchronization and thus, a higher percentage of cells are susceptible to the effects of a DNA synthesis inhibitor. Therefore, since asparaginase has been shown to inhibit DNA synthesis (24, 47, 66, 96, 148), the consequences of an equivalent degree of inhibition on more cells should result in greater cytotoxicity. This argument is based, however, on the assumption that asparaginase is an S-phase specific agent. In fact, the literature review above implies that the non-S-phase specific inhibition of membrane synthesis may be a more important component of asparaginase's mechanism of action than is asparaginase-induced inhibition of DNA synthesis (48, 117). In addition, an enhanced rate of DNA synthesis is not adequate evidence for the synchronization of tumor cells.

In an attempt to prove that ara-c synchronizes tumor cells in this tumor model and at this dosage, the labeling index and mitotic

index were determined and the results presented in Table VIII. A labeling index of 96% was observed for the first post-ara-c point which is consistent with virtually a pure S-phase population. Furthermore, the mitotic index for these cells 90 minutes post-wash was only 0.8% and after 6 hours, when the cells were able to traverse S-phase, the mitotic index reached a peak of 15.3%. However, this result is mitigated by a control labeling index of 75% suggesting that under these culture conditions the control cells spend three-fourths of the cell cycle in S-phase. In addition, the mitotic index for the ara-c pretreated cells never exceeds that of the control. This may, however, only imply that the peak mitotic index following ara-c occurred at a time different from the data points chosen. Even though the data from the mitotic and labeling indexes suggest cell synchronization with ara-c, it also implies that the L5178Y tumor model used in these experiments is an inadequate system from which to draw definite conclusions on the S-phase specificity of asparaginase. Since numerous investigators have demonstrated cell synchronization with ara-c (1, 8, 42, 85, 127, 159, 164), the investigation of the interactions of ara-c and asparaginase in another tumor model with a slower rate of growth might be of considerable value.

In addition to demonstrating cell synchronization, the autoradiographic data helps to clarify the mechanism by which ara-c causes cell death. Many studies as reviewed above have suggested that ara-c's cytotoxicity is the result of competitive DNA polymerase inhibition (51, 55, 69, 70, 101, 154). However, other studies have shown that

Table VII

L5178Y cells in culture were treated with ara-c 10^{-6}M for 8 hours and following the removal of the drug, the labelling index (autoradiograms) and mitotic index were determined as a function or time. Results represent the mean of 1000 cells counted.

Time Post Wash	Condition	Labelling Index	Mitotic Index
90 minutes	Control	80.8%	7.3%
	Ara-C	95.5%	0.8%
210 minutes	Control	84.0%	8.3%
	Ara-C	94.0%	3.6%
6 hours	Control	82.2%	15.9%
	Ara-C	82.1%	10.5%
8 hours	Control	77.9%	10.9%
	Ara-C	68.3%	15.3%
10 hours	Control	78.8%	12.3%
	Ara-C	85.5%	8.3%

cell damage induced by high doses of ara-c is either not reversed or only partially reversed by dCTP. This irreversible component of ara-c's action could result from failure of the DNA polymerase to recover following removal of the drug. However, the 96% labeling index following ara-c's removal shows that the DNA polymerases are functioning in virtually all cells and despite this, only 53% of the cells are viable by cloning (Table II). These results support the conclusions of others (32, 84, 165) that ara-c's cytotoxicity is not adequately explained by the observed competitive inhibition of DNA polymerases.

Since the data presented for the effect of asparaginase on DNA synthesis do not completely explain the observed synergistic response, the effect of ara-c on other macromolecules was investigated. Although it has been reported that ara-c does not inhibit protein synthesis directly (143), the effect of ara-c pretreatment on protein synthesis was investigated to rule out the possibility that ara-c potentiates the inhibition of protein synthesis by asparaginase. The effects of asparaginase on protein synthesis, 90 and 210 minutes after removal of ara-c, is presented in Table VIII. At 210 minutes, asparaginase caused 84.7% inhibition in both groups. Furthermore, the difference between 90% inhibition for the control and 77% following ara-c at 90 minutes is probably not significant, but it is difficult to draw conclusions based on a single experiment. However, considering both time points, it seems unlikely that ara-c profoundly effects asparaginase's inhibition of protein synthesis. Of course it is

Table VIII

Effect of post wash interval on H^3 leucine incorporation with and without asparaginase follwoing either saline or 10^{-6}M ara-c for 8 hours. Data represents a single experiment. Asn'ase=asparaginase 0.5 IU/ml. Rate=dpm/10^6 cells / minute incubation. % Control= rate of ara-c divided by the rate of control.

Time	Group	Rate	Rate with Asn'ase	% Inhibition	% Control
90 min.	Control	1798	176	90.2%	
	Ara-C	1868	433	76.8%	104%
210 min.	Control	1598	245	84.7%	
	Ara-C	2752	422	84.7%	172%

Table IX

Effect of asparaginase on H^3 ara-c incorporation into cold acid precipitalbe material. Data represents the mean of 4 experiments when either saline or asparaginase(0.5 IU/ml) was added at the beginning of a 4 hour incubation. Data is expressed as the mean rate $\pm$ 1 standard deviation. Rate=dpm/10^6 cells/minute incubation.

Saline	158 $\pm$ 11.7	
Asparaginase	60 $\pm$ 3.8	61.2% inhibition with asparaginase

impossible from this data to exclude an interaction on a specific protein.

In summary, the data presented for the effect of the combination of ara-c and asparaginase on DNA and protein synthesis does not substantiate the hypothesis that ara-c potentiates the biochemical effects of asparaginase. Therefore, an alternate theory must be proposed to explain why this combination is most effective in vitro when asparaginase is added to the cells within 90 minutes of ara-c's removal (Table III). The schedule dependency of this combination suggests favorable cytokinetic timing so that asparaginase's inhibition of protein synthesis occurs at a critical time to the cell. Furthermore, since the initiation of DNA synthesis requires protein synthesis (63, 121, 140), the process of recovering from ara-c's toxicity might be associated with an increased demand for protein synthesis. In fact, the results in Table VIII show that following ara-c, the rate of protein synthesis is 172% of control at 210 minutes. Thus in terms of cytotoxicity, these cells may be more sensitive to an equivalent degree of inhibition with asparaginase.

Further research could be directed at elucidating which specific proteins are required for cells to recover from ara-c and what are the effects of asparaginase on the synthesis of these proteins. Of particular interest would be the effect of this combination on the cellular levels of the α and β DNA polymerases. Since the α polymerase is required for DNA replication (11, 27, 28), inhibition of its synthesis by asparaginase would prevent the cells from reinitiating

cell growth following ara-c. Furthermore, if asparaginase inhibits the synthesis of the repair enzyme, β polymerase (160), the cells would be unable to excise the ara-c incorporated into DNA, thus potentiating the toxicity of ara-c. Therefore, if asparaginase inhibited the synthesis of either α or β DNA polymerase, it could explain the schedule-dependent synergy of this combination.

Another biochemical locus that might be affected by the combination of ara-c and asparaginase is histone synthesis. Histone synthesis is an S-phase event that is necessary for the assembly of newly-formed DNA complexes (131). Boren et al. have demonstrated a specific inhibition of histone messenger RNA and histone synthesis by ara-c (12, 135). Furthermore, since asparaginase inhibits protein synthesis (24, 47, 95, 96, 138, 147, 148, 163) probably including inhibition of histone synthesis, the combination might produce an additive or synergistic inhibition of histone synthesis. Confirmation of this hypothesis would require direct measurement of histone synthesis following this combination.

It is also possible that the synergistic cell kill produced by the sequential administration of ara-c and asparaginase results from an enhanced rate of RNA catabolism. Asparaginase might increase the destruction of RNA as a result of higher levels of ribonuclease, an enzyme that hydrolyses RNA. In fact, asparaginase has been reported to increase ribonuclease in murine lymphosarcoma P1798 (2). Furthermore, Boren et al. (12, 135) demonstrated that ara-c decreased the translation of 7-9 S mRNA. Therefore, the combination of enhanced destruction

and decreased translation of RNA would indeed be a serious cellular lesion. However, this mechanism for drug synergy is only hypothetical and further research is necessary to demonstrate that an enhanced turnover of RNA indeed occurs.

The biochemical studies presented above were undertaken to explain the observed additive or synergistic response of the sequential administration of ara-c followed by asparaginase, but they provide no explanation of the antagonism of the reverse combination. Therefore, a small group of experiments was done to try to understand the observed antagonism. Based on the hypothesis that ara-c's cytotoxicity was associated with the incorporation of ara-c into RNA and DNA, the effect of asparaginase treatment on ara-c's incorporation into TCA precipitable macromolecules was investigated. The method used gives only the sum of the incorporation of ara-c into RNA and DNA and does not differentiate between them. The results presented in Table IX confirm the findings of other investigators that ara-c is incorporated into macromolecules (29, 32, 38, 56, 143, 166), and that the incorporation is linear over 4 hours (Figure 7). Table IX also shows that asparaginase caused a 61% inhibition of ara-c's incorporation into macromolecules suggesting that this may be a mechanism of antagonism. This antagonism could result from asparaginase inhibiting the transport of ara-c into the cells as has been previously demonstrated in the L5178Y leukemia cells by Nahas and Capizzi (107). Following asparaginase treatment, they observed decreased intracellular concentrations of ara-c, methotrexate, 6-mercaptopurine

and 5-fluorouracil. They concluded that this effect is a nonspecific membrane defect that might result from asparaginase's inhibition of membrane glycoprotein synthesis. An alternate explanation for asparaginase's antagonism of ara-c's incorporation is that asparaginase's inhibition of cellular DNA synthesis prevents incorporation of this cytidine analog.

In order to differentiate between these two possible mechanisms of antagonism, asparaginase was added to the cells after two hours of incubation with H^3 ara-c, which is a time when the intracellular steady state concentration of ara-c should have already been reached. Therefore, if inhibition with asparaginase occurred after this addition, it could be assumed not to be associated with a transport defect. The observed change of slope of curve C in Figure 6 suggests that asparaginase directly inhibits ara-c incorporation into macromolecules independent of a membrane effect. However, this does not exclude the possibility that a membrane effect might coexist. This experiment represents only a preliminary study. Final proof that asparaginase antagonizes ara-c by preventing its incorporation into DNA would require the separation of the effects of asparaginase on the incorporation of ara-c into DNA and RNA and demonstrating that the intracellular pool of ara-c is not effected by asparaginase.

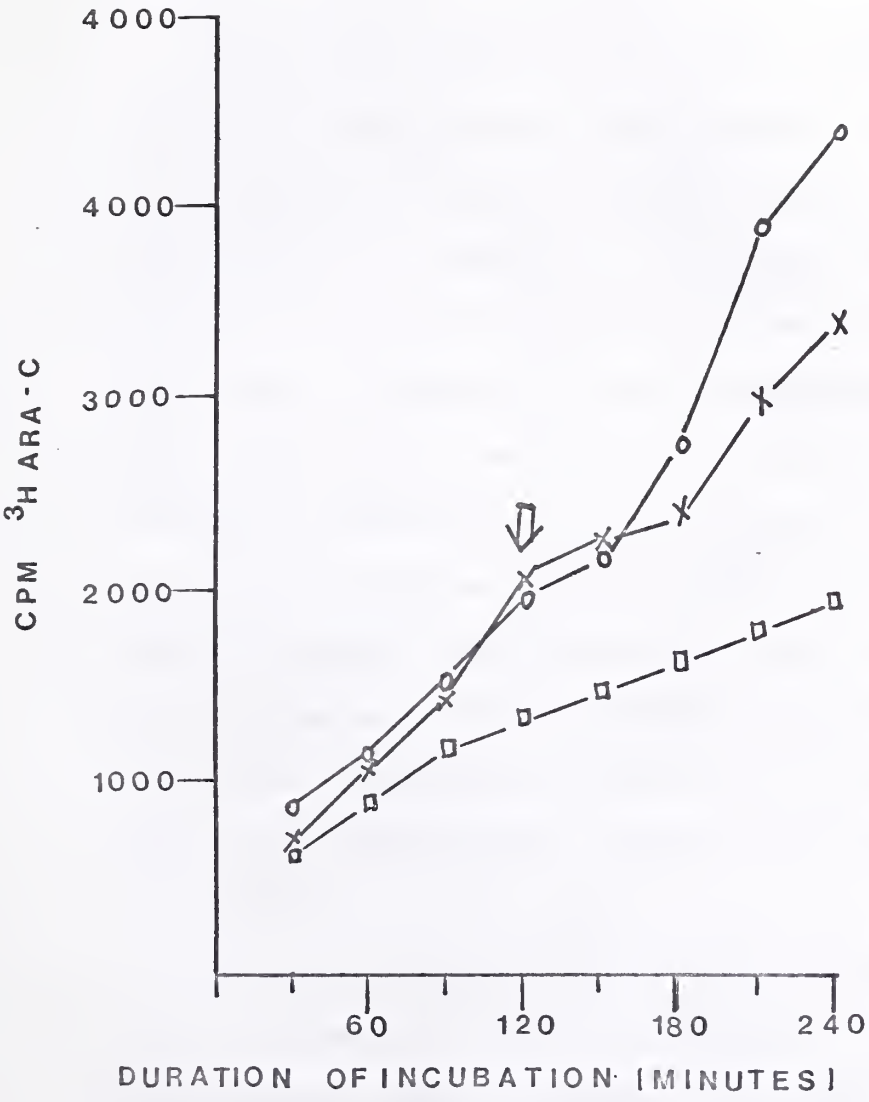


Figure 7:Effect of asparaginase on H^3ara-c incorporation into cold acid precipitable material. Data points represent the average of two experiments. (0)=control. (□)=asparaginase at time 0. (X)=asparaginase at 120 minutes as indicated by arrow.

Summary

The aim of this thesis project was to determine if the combination of ara-c and asparaginase shows schedule-dependent synergy and/or antagonism in the treatment of murine leukemia L5178Y. Prior to investigating this possibility, *in vivo* tumor growth curves were determined as a basis for selecting treatment schedules. These studies showed that following either ara-c or asparaginase, the tumor burden rapidly falls and does not begin to recover until 120 hours post-treatment. Consequently, a 120 hour interval was chosen between drugs for the sequential treatment schedules in the animal survival studies. The mouse studies demonstrated that the sequential use of ara-c and then asparaginase resulted in synergistic animal survival. However, when asparaginase preceded ara-c or when both drugs were administered simultaneously, a subadditive or antagonistic response was observed.

The schedule-dependent synergy and antagonism observed *in vivo* for the combination of ara-c and asparaginase was investigated further *in vitro* by cell viability studies. As before, an antagonistic response was observed for the sequential use of asparaginase and then ara-c and for the simultaneous treatment with both drugs. Furthermore, an additive, if not synergistic, response occurred following the sequential treatment with ara-c and then asparaginase, but this favorable

response was lost when the interval between drugs exceeded 90 minutes. The observation confirms the *in vivo* data and supports the importance of understanding drug-drug interactions.

In an attempt to explain the observed synergy, the effect of the sequential administration of ara-c and then asparaginase on macromolecular synthesis *in vitro* was explored. Ara-c pretreatment did not potentiate the asparaginase-induced inhibition of DNA synthesis. Furthermore, this lack of potentiation was not influenced by different doses of ara-c or by the time interval betewen ara-c and asparaginase. In addition, ara-c did not potentiate the effect of asparaginase on protein synthesis.

An enhanced rate of DNA and protein synthesis, as compared to control, was observed 210 minutes after ara-c's removal. This observation is consistent with ara-c induced partial cell synchronization and is supported in part by the results of the labeling and mitotic indices. However, in this tumor model the increase in S-phase cells following ara-c is too small to account for the synergistic effect of the combination. Although no definitive mechanism can be proposed to explain the observed synergistic response, it seems most likely that asparaginase is preventing the tumor cells from recovering from ara-c's toxicity. Further implications of this and other mechanisms are discussed.

Finally, data is presented that shows that asparaginase prevents ara-c's incorporation into macromolecules. Since ara-c's incorporation into macromolecules is associated with its cytotoxicity,

inhibition of its incorporation could explain the antagonism of the sequential use of asparaginase and then ara-c.

It is hoped that the use of this data to devise a new treatment protocol for acute leukemia will result in an enhanced therapeutic response and in the prolongation of a human life.

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